Failure to elicit seroresponses to pneumococcal surface proteins (pneumococcal histidine triad D, pneumococcal choline-binding protein A, and serine proteinase precursor A) in children with pneumococcal bacteraemia

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Abstract

Pneumococcal surface proteins (PSPs) elicit antibody responses in infants and young children exposed to *Streptococcus pneumoniae*. These seroresponses could contribute to the aetiological diagnosis of pneumococcal disease, e.g. during the clinical development of novel PSP-based vaccines. In this study, we assessed the kinetics of antibody responses to three highly conserved and immunogenic PSPs (pneumococcal histidine triad D (PhtD), pneumococcal choline-binding protein A (PcpA), and serine proteinase precursor A (PrtA)) in 106 children (median age, 21.3 months; males, 58.5%) admitted for pneumococcal bacteraemia. Anti-PhtD, anti-PcpA and anti-PrtA antibodies were measured by ELISA, and compared in 61 pairs of acute (≤7 days) and convalescent (>14 days of admission) serum samples. Acute serum titres were similar to those observed in healthy children, and were unaffected by the acid dissociation of circulating immune complexes. Despite proven bacteraemia, seroresponses (≥2-fold increase in anti-PSP antibody concentrations) were only identified in 31 of 61 children (50.8%), directed against PrtA (n = 23, 37.7%), PcpA (n = 19, 31.1%), and PhtD (n = 16, 26.2%), or several PSPs (two PSPs, n = 13, 21.3%; three PSPs, n = 7, 11.5%). Certain seroresponses were very strong (maximal fold-increases: PhtD, 26; PcpA, 72; PrtA, 12). However, anti-PSP antibody concentrations failed to increase in the convalescent sera of 30 of 61 (49.2%) bacteraemic children, and even declined (≥2 fold) in 13 of 61 (21.3%), mostly infants aged <6 months (8/13, 61.5%), possibly through consumption of maternal antibodies. Thus, pneumococcal bacteraemia may fail to elicit antibody responses, and may even have an antibody-depleting effect in infants. This novel observation identifies an important limitation of serology-based studies for the identification of bacteraemic children.

Keywords: Antibodies, bacteraemia, children, *Streptococcus pneumoniae*, surface proteins
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Introduction

Streptococcus pneumoniae is a common pathogen causing invasive diseases, and an important cause of paediatric mortality [1–3]. Pneumococcal bacteraemia continues to be of concern, because of its inherent risk of progressing to sepsis or meningitis in young children [4]. Its diagnosis is often challenging, as blood cultures may be unavailable or negative [4]. Although conjugated pneumococcal vaccines have decreased its incidence [1,4–8], there are concerns related to the emergence of non-vaccine *S. pneumoniae* serotypes [3]. New strategies are thus being investigated to develop vaccines that include pneumococcal surface proteins (PSPs) as antigens for 'universal' pneumococcal vaccines [9,10]. Specific antibodies against PSPs are produced

naturally [11], and infants may already develop anti-PSP antibodies in response to *S. pneumoniae* colonization (Hagerman A, Posfay-Barbe KM, Grillet S, Ochs MM, Brookes RH, Greenberg D, Givon-Lavi N, Dagan R, Siegrist C-A, unpublished data) or infection. As some of these PSPs are conserved in >97% of *S. pneumoniae* serotypes [12], PSP-specific seroresponses could be used for the aetiological confirmation of suspected *S. pneumoniae* infections, e.g. during vaccine studies. Three surface-exposed PSPs were selected primarily on the basis of their availability, their putative role(s) in bacterial pathogenesis, and their conservation across pneumococcal strains (>97%) [12]. Pneumococcal histidine triad D (PhtD) is thought to be involved in the invasion process [13]. Anti-PhtD antibodies have been detected in convalescent-phase sera in children with pneumococcal bacteraemia, confirming that this protein is exposed and recognized by the immune system during pneumococcal disease [13,14]. Pneumococcal choline-binding protein A (PcpA) is a choline-binding protein that is suggested to play a role in bacterial adherence to the epithelium in the lower respiratory tract. PcpA is distinct from other pneumococcal choline-binding proteins (PspC and CbpA) [13,15,16]. Some studies have shown that the manganese concentration directly affects the surface expression of PcpA, its expression probably being increased when bacteria infect low-manganese sites, such as lungs and blood [15]. Serine proteinase precursor A (PrtA) contributes to pneumococcal virulence [11], and its expression is also regulated by manganese concentrations.

The aim of this study was to define the kinetics of antibody responses to three immunogenic and conserved PSPs (PhtD, PcpA, and PrtA) in children with proven pneumococcal bacteraemia. We compared two populations, living in the same region with different socio-economic conditions and lifestyles, and evaluated the influence of age and ethnicity on these seroresponses.

Methods

Patients

Children <15 years of age were enrolled prospectively. They were admitted to the Soroka University Medical Centre, Israel between 1996 and 2007 with proven bacteraemia (positive blood culture for *S. pneumoniae*), and provided paired acute/convalescent blood samples after written informed consent had been obtained. None had been immunized against *S. pneumoniae*. Serum samples were stored at −70°C until analysis. The study was approved by the Soroka University Medical Centre Ethics Committee. Acute samples were obtained within 7 days of admission, and convalescent samples were obtained at least 2 weeks after admission.

Setting

In southern Israel, the Jewish population is mainly urban, whereas the Bedouin population is gradually moving away from its nomadic lifestyle [17]. Children of the two populations do not frequent the same day-care facilities or schools, and do not have a common social life, but all are entitled to free medical care. In 2004, the crude birth rate was 55.3 vs. 21.0 births per 1000 persons in the Bedouin and Jewish populations, respectively [18], and the mean ± standard deviation family size among the Bedouin population was 8.2 ± 0.9 persons as compared with 3.2 ± 0.1 persons among the Jewish population [19]. Hospitalization rates for respiratory and other infectious diseases were higher among Bedouins [20]. During the study period, the seven-valent pneumococcal conjugate vaccine had not yet been introduced in Israel, and <3% of the population had participated in clinical trials with one of the experimental pneumococcal conjugate vaccines.

PhtD, a truncated version of PcpA and a truncated histidine-tagged version of PrtA were recombinantly expressed in and purified from *Escherichia coli*. The specificity of PhtD and PcpA antigen-specific ELISA was demonstrated by complete loss of binding of human antibody sera by competitive inhibition with specific antigens, and not with other PSPs as control antigens.

Serological analyses

Paired acute and convalescent serum samples were tested in the same run by indirect ELISA, with purified proteins being used to coat Immulon (Thermo Labsystem, Helsinki, Finland) plates, anti-IgG antibody (Cappel Laboratory, Cochranville, PA, USA), and ABTS substrate, as previously described [12]. Eight serial dilutions (two-fold) of serum samples were performed to allow precise quantification of antibody titres. A common reference human antibody serum was used in each assay. Its antibody concentration in ELISA Units was defined by the reciprocal of its dilution at OD = 1.0. Assay results were expressed in EU/mL by interpolation with the reference serum, allowing precise quantification. A cut-off of 5 EU/mL was experimentally identified as allowing the reliable detection of serum concentrations. Anti-PSP antibody concentrations below 5 EU/mL were given a titre of 2.5 EU/mL. A significant rise in antibody titres was conservatively predefined as a minimal two-fold (100%) increase between the acute-phase and convalescent-phase samples.

To determine whether low antibody concentrations reflected the presence of circulating immune complexes, the sera of 35 children aged ≥12 months were acid-treated by incubation in a 0.2 M glycine-HCl (pH 2.5) dissociation buffer for 20 min at room temperature [21]. A Tris-HCl 1 M

(pH 9.5) buffer was added to stop the dissociation reaction, immediately prior to the parallel quantification of serum antibodies in dissociated/native sera to identify samples with enhanced ELISA signals.

Statistical analyses

Statistical analysis was carried out with SPSS (PASW Statistics 18.0.0; IBM Corporation, Somers, NY, USA). Standard descriptive statistics were used to describe socio-demographic characteristics. Categorical data were compared by the use of chi-square tests or Fisher's exact tests when appropriate. Serological findings among groups were compared by using Student's *t*-test, linear regression, or the Mann–Whitney *U*-test, according to the type of independent variable. A multivariate linear regression analysis model was used to assess goodness-of-fit and calculate adjusted ORs and 95% CIs, for variables that had p-value of <0.25 in univariate analyses. For all statistical tests, differences were considered significant at $p < 0.05$ or when the 95% CI did not include 1.0.

Results

One hundred and six children aged 7 days to 15 years were enrolled. They were divided in five age groups, each including at least 17 children (Table 1). Bacteraemic children were more frequently from the Bedouin (70.8%) community, and were more likely to be boys (58.5%).

Acute serum samples were available for 95 children. Antibodies against all three PSPs were detectable in all age groups (Table 2a), ranging from very low to high levels. The lowest anti-PSP antibody concentrations were observed in infants, and the highest in children aged >24 months (PcpA) or 47 months (PhtD and PrtA). In contrast to healthy children from the same communities (Hagerman A. *et al.*, unpublished data), anti-PSP antibody concentrations were not higher in Bedouin children overall. The dissociation of circulating immune complexes by acid treatment did not increase serum antibody concentrations (mean ratios in acid-treated/naïve samples: PhtD, 0.96 ± 0.18; PcpA, 0.99 ± 0.09; PrtA, 0.85 ± 0.17).

Convalescent serum samples were available for 72 children. Anti-PSP antibodies were detected in all children, again at similar concentrations in the two ethnic groups (Table 2b). Seroresponses to pneumococcal bacteraemia were measured after a median interval of 30.6 days in 61 children with available paired acute/convalescent samples (Table 3). This sampling interval did not affect antibody responses (data not shown). The mean convalescent/acute geometric mean concentration ratios were PSP-specific (PhtD, 2.32; PcpA, 4.62; PrtA, 2.28) (Fig. 1). The maximal ratio was high for each PSP (anti-PhtD, 26-fold; anti-PcpA, 72-fold; anti-PrtA, 12-fold), confirming the immunogenicity of these three PSPs. However, seroresponses (≥2-fold increase) were observed in only 31 of 61 (50.8%) children, regardless of age (Fig. 2). Antibodies were directed against PrtA, PcpA and PhtD in, respectively, 23 (37.7%), 19 (31.1%) and 16 (26.2%) children. Seroresponses were observed against two PSPs in 13 (21.3%) children and against all three PSPs in seven (11.5%) children.

In three bacteraemic children, a >2-fold decline in the concentration of antibody against a specific PSP was observed, in parallel with a ≥2-fold increase in the concentration of antibody against another PSP. Lower anti-PSP antibody concentrations in convalescent than in acute samples were observed in 13 of 61 (21.3%) children, mostly in infants younger than 6 months (8/13, 61.5%). This decline in anti-PSP antibody concentrations affected two or three PSPs in seven of 13 (53.8%) children, confirming their biological significance.

Remarkably, 30 of 61 (49.2%) children failed to raise even a modest (≥2-fold) seroresponse to any of the three immunogenic and conserved PSPs tested. Among these non-responders, 20 (66.7%) failed to increase their antibody concentrations above baseline values, whereas a decline in anti-PSP antibody

TABLE 1. Characteristics of 106 children with pneumococcal bacteraemia

	Age group	Total *n* (median, IQR)	Bedouin children, *n* (%)[a]	Jewish children, *n* (%)[a]
Age group distribution in months	All	106 (21.3, 43.9)	75 (70.8)	31 (29.2)
	0–5[b]	21 (3, 2.9)	18 (24)	3 (9.7)
	6–11[b]	17 (9, 4.4)	13 (17.3)	4 (12.9)
	12–23[b]	20 (18.6, 6.2)	14 (18.7)	6 (19.35)
	24–47[b]	20 (34.8, 15)	14 (18.7)	6 (19.35)
	>47[b]	28 (90.5, 72)	16 (21.3)	12 (38.7)
Male gender, *n* (%)		62 (58.5)	46 (61.3)	16 (51.6)

IQR, interquartile range.
[a]All comparisons not statistically significant.
[b]Percentage within ethnicity.

TABLE 2. Anti-pneumococcal surface protein antibody geometric mean concentrations (GMC) of children with acute and convalescent blood samples following bacteriologically proven pneumococcal bacteraemia, by age group and ethnicity

		GMC (EU/mL)			
Age group (months)	***n***	**All (95% CI)**	**Bedouin children**	**Jewish children**	**p-value**
(a) Acute blood samples					
PhtD					
All	95	37.6 (26.2–54)	33.4	55.5	NS
0–5	19	16.5 (7.4–36.3)	14.7	42.7	NS
6–11	15	11.2 (4.8–26)	10.7	13.2	NS
12–23	18	20.8 (9.5–45.7)	33.3	4.1	0.049
24–47	17	65.5 (33.3–128.7)	64	70.7	NS
>47	26	145.5 (94.1–225.2)	119.4	183.3	NS
PcpA					
All	95	316.3 (227.5–439.8)	293.6	394.6	NS
0–5	19	181.8 (96.2–343.7)	180.3	195	NS
6–11	15	131 (41.8–410.2)	121.5	176.9	NS
12–23	18	148.1 (75.7–289.6)	170.7	90.1	NS
24–47	17	707.4 (399.2–1253.4)	1025	211.9	0.031
>47	26	788.2 (535–1161)	596.3	1091.3	NS
PrtA					
All	95	70.3 (47.3–104.4)	70.8	73.2	NS
0–5	19	41.8 (20.5–85.2)	38.2	89.4	NS
6–11	15	13.5 (6–30.4)	12.9	16.3	NS
12–23	18	22.6 (10.6–48.4)	39.2	3.3	0.009
24–47	17	139.7 (66.1–295.4)	178.9	62.6	NS
>47	26	372.2 (219.5–631)	442.2	304.4	NS
(b) Convalescent blood samples					
PhtD					
All	72	56.5 (36.1–88.3)	45.6	103.1	NS
0–5	15	17.2 (7.9–37.5)	17.7	15.3	NS
6–11	12	15.8 (4.7–52.6)	15.3	18.7	NS
12–23	14	45.5 (20–103.6)	39.6	64.3	NS
24–47	8	123.8 (24.2–635)	97	186	NS
>47	23	207.2 (131.5–326.5)	167.6	288.1	NS
PcpA					
All	72	438.7 (287.3–669.6)	452.7	415.4	NS
0–5	15	134.1 (50.5–356.2)	181.6	39.9	NS
6–11	12	402.9 (112.8–1438.8)	363.9	670.5	NS
12–23	14	296.9 (123.3–714.9)	356.1	188.4	NS
24–47	8	771.3 (215.4–2761.4)	1529.7	246.3	NS
>47	23	1035.3 (657.5–1630.1)	860.8	1379.6	NS
PrtA					
All	72	99.8 (62.1–160.5)	112.4	85.3	NS
0–5	15	29.5 (15.2–57.3)	32.1	21.1	NS
6–11	12	19.4 (7.5–50.1)	18.5	24.5	NS
12–23	14	62.4 (23.8–163.7)	114	13.8	NS
24–47	8	342.2 (104.2–1123.4)	346.9	334.5	NS
>47	23	450.6 (231.6–876.6)	648.9	255.4	NS

NS, not significant; PcpA, pneumococcal choline-binding protein A; PhtD, pneumococcal histidine triad D; PrtA, serine proteinase precursor A.

concentration between acute and convalescent samples was observed in ten (33.3%) children. There was no significant influence of age or ethnicity on seroresponses.

Discussion

This study identifies a major hurdle to the potential use of seroresponses for the aetiological diagnosis of *S. pneumoniae* pneumococcal bacteraemia: a bacteriologically proven pneumococcal infection may fail to elicit antibody responses to highly conserved PSPs, both in infants and in toddlers. Although the pattern and the magnitude of seroresponses differed between PhtD, PcpA, and PrtA, they followed similar trends.

Whether children admitted for pneumococcal bacteraemia had lower anti-PSP antibody titres than healthy children in the same age group and community is as yet unknown. Indeed, certain anti-PSP antibodies are protective against murine invasive disease [10,14,15,22], and anti-PSP seroresponses could contribute to the decline in the incidence of pneumococcal invasive disease after the second year of life [23]. In support of this hypothesis, Obaro *et al.* [24] showed lower anti-PSP antibody titres in colonized than in uncolonized infants, raising the possibility that some anti-PSP antibodies may protect against *S. pneumoniae* carriage and therefore against invasive disease. Our group previously reported three-fold lower concentrations of anti-PcpA antibodies in the acute sera of children diagnosed with community-acquired pneumonia of presumed pneumococcal

TABLE 3. Anti-pneumococcal surface protein antibody geometric mean concentrations (GMCs) of 61 children with paired acute and convalescent blood samples following bacteriologically proven pneumococcal bacteraemia, by age group

Age group (months)	*n*	Mean GMC (EU/mL)	
		Acute sample	Convalescent sample
PhtD			
All	61[a]	137.3	217.9
0–5	13	50.08	62.46
6–11	9	46.89	56.44
12–23	12	47.25	85.75
24–47	5	118.4	608.8
>47	21	286.62	364.33
PcpA			
All	61[a]	732.2	1370.5
0–5	13	389.77	879.62
6–11	9	916.44	1673.44
12–23	12	269.17	690.25
24–47	5	1020	1631.8
>47	21	1077.05	1902.14
PrtA			
All	61[a]	276.2	493.9
0–5	13	87.23	56.54
6–11	9	70.22	70.56
12–23	15	76.75	218.67
24–47	5	220.6	482.4
>47	21	606.62	1044

PcpA, pneumococcal choline-binding protein A; PhtD, pneumococcal histidine triad D; PrtA, serine proteinase precursor A.
[a]One patient: age missing.

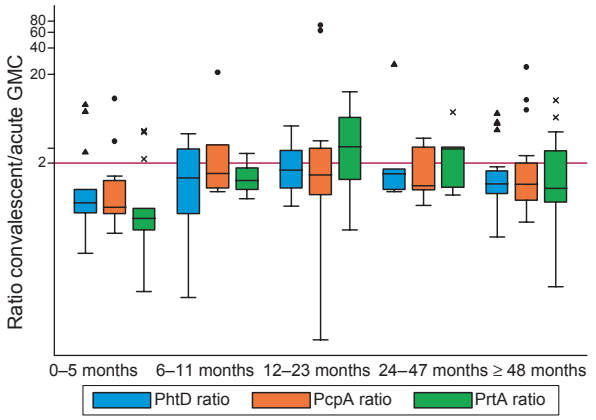

FIG. 1. Fold-increase of anti-pneumococcal surface protein (PSP) antibody concentrations in bacteraemic children. The ratio of convalescent to acute anti-PSP antibody geometric mean concentration (GMC) is illustrated for each PSP and age group. PcpA, pneumococcal choline-binding protein A; PhtD, pneumococcal histidine triad D; PrtA, serine proteinase precursor A.

origin in Geneva, Switzerland [12], and regression analyses identified a low acute anti-PcpA antibody concentration as an independent predictor (p 0.002) of community-acquired pneumonia of presumed pneumococcal origin. This pattern of lower anti-PcpA antibody concentrations at admission was

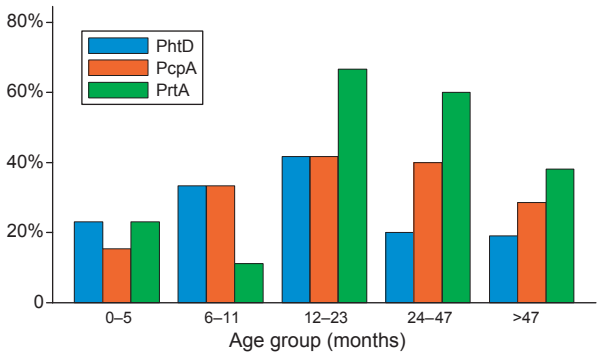

FIG. 2. Proportion of seroresponders among bacteraemic children by age category. The proportion of bacteraemic children with a ≥2-fold increase in anti-PSP antibody concentration is illustrated for each pneumococcal surface protein and age group. PcpA, pneumococcal choline-binding protein A; PhtD, pneumococcal histidine triad D; PrtA, serine proteinase precursor A.

not observed in Israeli children with pneumococcal bacteraemia: anti-PSP antibody concentrations in bacteraemic children were similar to or higher than those in healthy children in both communities (Hagerman A. *et al.*, unpublished data). This is in accordance with a report including 25 adult patients with pneumococcal meningitis [11]. An exception was observed between 12 and 23 months, anti-PSP antibody titres being significantly lower in bacteraemic than healthy children (geometric mean concentrations: PhtD, 72.3 vs. 20.8, p 0.023; PcpA, 813.7 vs. 148.1, p 0.004; PrtA, 95.2 vs. 22.6, p 0.022). Whether low anti-PSP antibody concentrations predispose toddlers to bacteraemic infections should thus be assessed in longitudinal prospective cohort studies.

Approximately half of the bacteraemic children raised significant seroresponses in the weeks following the initial (acute) visit. These anti-PSP responses were occasionally very strong, as reported in 14 adults [25]. They were directed against one (26–38%), two (21.3%) or even three (11.5%) of the three PSPs tested. This confirmed both their surface expression by the invading pathogens and their strong immunogenicity. The strongest seroresponses were raised to PcpA (72-fold) and PrtA (12-fold). This may reflect the triggering of PcpA/PrtA expression by the lower-manganese (Mn^{2+}) environment during bacteraemia than in the nasopharynx [15,26].

Unexpectedly, such seroresponses were absent in half of the children with confirmed pneumococcal bacteraemia. Several hypotheses may explain this high frequency of non-responders. Anti-PSP antibodies produced against a single pathogenic protein may not recognize all allelic variants [10], although these PSPs are highly conserved [12]. Nevertheless, genes may be absent or not expressed in 1–3% of

the strains. However, this study was performed over a period of several years, and it therefore seems highly unlikely that a large clonal expansion of PSP-negative strains can explain these results. Finally, the high diversity of serotypes found among these children also makes it unlikely that certain PSPs were 'hidden' by, for example, the polysaccharidic capsule. Young infants may not raise seroresponses, because of their immune immaturity and/or the inhibitory influence of maternal antibodies [27], and, indeed, infants aged <6 months were over-represented among non-responders. It may be difficult for older children with high pre-existing antibody concentrations, or whose antibody responses were initiated a few days prior to admission, to further increase their antibody levels ≥2-fold. Specifically regarding the children with the highest acute concentrations of antibodies against these three PSPs, this group of children was less likely to have a ≥2-fold antibody increase over time, although this was only significant for PhtD (data not shown). However, the concentrations of antibodies against at least one of the three PSP tested were frequently low, which should have allowed the detection of seroresponses. The presence of circulating immune complexes may reduce the concentrations of antibodies against pneumococcal polysaccharides, which may also affect anti-PSP antibodies. Lower convalescent than acute anti-PSP antibody titres were, indeed, occasionally observed, mostly in infants younger than 6 months of age, in whom baseline anti-PSP antibodies essentially reflect passively transmitted maternal antibodies [13], and in whom convalescent titres may fail to increase, as a result of immune immaturity and/or the inhibitory influence of maternal antibodies (reviewed in [27]). However, the dissociation of immune complexes remained without influence on anti-PSP antibody concentrations, which is likely to reflect the greater abundance of circulating polysaccharides than of specific PSPs. Seroresponses may take more than 2–4 weeks to reach significant levels, and may thus have been missed by the timing (mean, 30.6 days) of our convalescent blood sampling. However, we observed no increase in anti-PSP seroresponses with time after admission, and infection-induced antibody concentrations tend to decrease during the late convalescent phase [11]. We cannot exclude the possibility that adding many more PSPs would increase the likelihood of detection of significant seroresponses. As an alternative hypothesis, the pneumococcal bacteraemia may be too sudden to trigger significant seroresponses and/or elicit some type of transient 'immune paralysis'. This would be reminiscent of the observation that invasive pneumococcal disease [28] or even nasopharyngeal carriage [29] can result in hyporesponsiveness to the infecting serotype following subsequent immunization with a glycoconjugate vaccine [28], an as yet unexplained phenomenon.

Our study has limitations. We assessed only IgG antibodies, and cannot exclude the possibility that IgM or IgA patterns would be distinct. Although anti-PSP IgG responses were compared with those of healthy children of the same community measured during the same period in a parallel study, we could not match bacteraemic cases and controls to formally demonstrate similar anti-PSP antibodies at time of admission in healthy and bacteraemic children. We can also not exclude the possibility that antibody responses were initiated a few days before the diagnosis of the bacteraemic episode, as a result of recent pneumococcal colonization [30], or that seroresponses would have been detected at subsequent time-points.

In conclusion, pneumococcal bacteraemia may be associated with strong anti-PSP seroresponses, but may fail to increase anti-PSP antibody concentrations in half of the patients, or even have an antibody-depleting effect in young infants. This identifies an important limitation to the use of seroresponses for the identification of bacteraemic children during clinical trials and vaccine studies.

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Transparency Declaration

A. Hagerman, S. Grillet, D. Greenberg, N. Givon-Lavi and K. Posfay-Barbe have nothing to declare. M. Ochs and R. Brookes are employees of sanofi Pasteur, and have no other disclosure or conflict of interest to report. R. Dagan has received, in the last 5 years, grants/research support from Berna/Crucell, Wyeth/Pfizer, MSD, and Protea. R. Dagan has been a scientific consultant for Berna/Crucell, GlaxoSmithKline, Novartis, Wyeth/Pfizer, Protea, and MSD. R. Dagan was a speaker for Berna/Crucell, GlaxoSmithKline, and Wyeth/Pfizer. R. Dagan is a shareholder of Protea. C.-A. Siegrist has

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